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BIOLOGICAL BULLETIN

ON THE SUPERPOSITION OF FERTILIZATION ON PARTHENOGENESIS.

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CONTENTS.

I. INTRODUCTION.

The generally accepted statement that an egg once fertilized cannot be refertilized seems to be challenged by recent workers who maintain that artificial parthenogenesis may be followed by fertilization. There is evidently an inconsistency here involved that may be eliminated if the facts are known. The ideas of these different writers are considered later in this paper.

Various workers in contributing to the analysis of the problem of fertilization have presented many and diverse points of attack. One method or group of methods that has produced very striking results is that of initiation of development by known chemical agents, largely developed through the excellent works of Loeb in his studies on artificial parthenogenesis.

Lillie ('13 and '14) has discovered and described a specific sperm agglutinating substance secreted by the eggs of two marine forms (*Arbacia punctulata* and *Nereis limbata*). This substance is produced by the eggs of these forms at the time of rupture of the germinal vesicle and is liberated into the sea water, in which eggs have been standing for a short while: it escapes from the eggs in amounts readily detectable by using a sperm suspension of the same species as an indicator. Sperm are agglutinated in masses that break apart after a longer or shorter time; the reaction is reversible. To this agglutinating substance he has given the name fertilizin.

The nature of this substance is very little known but a number of its properties have been determined by Lillie and later Glaser ('14) has offered certain information concerning it. Of interest at present however is its rôle in the process of fertilization.

Fertilizin according to Lillie is always present in eggs when fertilization is possible, and so far experiments have shown it present in quantities sufficiently large to be detected by its sperm agglutinating properties. It is not detectable in immature eggs, likewise it is absent or bound immediately, or within a very short time, following the act of fertilization. In these two cases then there is a correlation between the presence of fertilizin and the capacity for fertilization; indeed Lillie has extended this conception to include all conditions of an egg—when fertilization is possible fertilizin is present. He holds that fertilization is a reaction or set of reactions in which this ovogenous substance fertilizin is activated: it is then "the effective agent which is transformed from an inactive to an active state by some substance in the spermatozoön."

The fertilizhin ypothesis attempts also to explain the mechanism of artificial parthenogenesis by assuming that development in this case is effected or initiated in essentially the same manner,

i. e., by the activation of this substance, within the egg. If this activation by parthenogenetic agents is rendered complete we should naturally expect the egg to acquire essentially the same condition as it does following the act of fertilization. If fertilization depends upon the activation of fertilizin and this has been once activated by artificial agents we should expect fertilization to be just as impossible as in a normally fertilized egg. Experiments lead us to believe that fertilization is a complex series of reactions which if once completed exclude all possibilities of repetition.

According to Loeb ('13, page 225) fertilization depends upon the introduction of two separate and distinct sperm-borne substances—a lysin-like substance and a substance producing the so-called corrective effect. The former is thought of as exercising a cytolytic influence upon the cortex of the egg that results in the production of a membrane: unless the influence of this lysin-like substance is counteracted in some way the processes initiated by it continue until the egg is totally destroyed. This substance acts before penetration of the sperm and is not specific, as shown by the fact that starfish sperm so act upon sea urchin eggs that they cause membrane production and subsequent destruction of the egg unless this is prevented by a secondary corrective agent. The latter substance according to Loeb acts only after the spermatozoön enters the egg; it stops the cytolytic action of the first substance and permits the egg to develop normally.

Loeb ('14 and '15) has objected to the fertilizin hypothesis on grounds which if correct, would go a long way toward sealing the fate of that hypothesis. Loeb's contentions in brief are these:

1. He maintains that this agglutinative substance is not a secretion from the egg but is derived from the clear transparent layer of jelly surrounding the egg, the so-called "chorion layer." If the jelly layer is destroyed no more fertilizin is present. Lillie ('14 and '15*a*) however has shown that this is not so.

2. He further asserts that eggs that have produced membranes as a result of butyric acid treatment or other parthenogenetic agents undergo not only further development but entirely normal

development if subsequently treated with sperm, provided only that the membranes are destroyed sufficiently to allow sperm to reach the eggs. In his opinion only those physical factors that may prevent sperm from coming in contact with the egg are concerned in the non-fertilizable character of eggs possessing distinct membranes.

His views are then entirely at variance with the idea that the egg bears its own fertilizing substance which is set in motion by activating agents, spermatozoa or artificial agents, and that once this activation has been effected no repetition of the same is possible. He leaves out of account any physiological change in the protoplasm of the egg and bases his entire conception upon purely physical factors at the boundary of the egg.

A more thorough analysis of the physiological conditions within the egg, following activation by any means, is highly desirable whatever theory we may be inclined to favor. One method of attack of such a problem is a more thorough study of the possibilities of a combination of parthenogenesis and fertilization. Certain observations have already been incidentally presented from this point of view.

Loeb ('13) finds that eggs of the sea urchin may be readily fertilized after membrane production if only the membranes are torn by shaking the eggs and sperm is added.

Herbst ('06) fertilized *Sphærechinus* eggs, having been previously treated with 50 c.c. sea water + 3 c.c. *n*/10 acetic acid for two to six minutes, with *Strongylocentrotus* sperm and obtained larvæ bearing predominant characters of the female parent. In the fertilization process membranes were not produced, cleavage was very abnormal, and resulting plutei were weak: there was usually a high degree of mortality in his cultures.

Tennent and Hogue ('06) were able to fertilize starfish eggs for a short time only, following membrane production induced by CO_2: according to their account sperm swam through the enlarged vitelline membrane entered the egg and caused the production of a second membrane.

Lillie ('14) calls attention to his observations that eggs of the sea urchin following membrane production are incapable of being fertilized.

The fundamental questions arising in consideration of these and other factors of fertilization are very evident. They aim at the very heart of the problem. It is necessary that we shall not lose sight of the distinction between penetration of the egg by the spermatozoön and the fertilization reaction; it is with the latter that we are more intimately concerned at this time. What then are the conditions within the egg that permit or prohibit fertilization? Is a reactivation of the egg or substances within the egg possible by the use of a different activator or can fertilization be superposed on parthenogenesis?

This problem was suggested to me by Professor Lillie and the experimental part was conducted under his supervision during the summers of 1914–15 at Woods Hole, Mass. It gives me great pleasure to express my indebtedness to him for his many suggestions and kindly criticisms during the progress of the work and also for a table in the Marine Biological laboratory. The examination of Preserved Material was conducted in the Hull zoölogical laboratory at the University of Chicago.

II. Material and Methods.

The writer's observations have been confined entirely to the Atlantic sea urchin (*Arbacia punctulata*). Fresh material from the live car was received daily and kept in aquaria of running sea water. Eggs for the experiments were obtained in the usual manner by cutting around the edge of the leathery oral disc and removing the ovaries entire to a clean finger bowl. The ovaries were cut up and fresh sea water added and the whole poured through cheese cloth. This allows the eggs to pass through, and retains on the filter the pieces of ovarian tissue. When the eggs had settled to the bottom of the dish the supernatant sea water was poured off and replaced by fresh sea water (200 c.c.–300 c.c.). This process of washing was usually repeated three times and eggs were removed from the stock for the individual experiments. For every experiment a control was also set aside.

Sperm ordinarily was easily obtained by allowing the males to shed the solid sperm into clean dry Syracuse dishes. This solid or "dry" sperm served as a stock supply from which the suspensions were made as needed.

Ordinary laboratory precautions were observed, such as sterilization of each urchin, hands, and instruments with tap water.

III. Combination of Parthenogenesis and Fertilization.

A great variety of agents, chemical as well as physical, have been found to be effective in initiating changes within the egg leading to development or partial development. Most of the agents lead to an imitation of the action of the spermatozoön in that cortical changes of the egg are induced by them that result in membrane production. The exception to the general rule seems to be the action of hypertonic sea water and even here accounts vary as to whether or not there is a cortical change involved. Membrane production has been recorded following exposure of sea-urchin eggs to saponin, distilled water, lipoid solvents, blood serum, fatty acids and other agents; but more consistent results have been obtained by the use of butyric acid than of any other agent and it is largely this method that has been employed in this study.

1. *The Curve of Fertilization after Butyric Acid Treatment.*

A certain well-known parallelism exists between eggs fertilized by sperm and eggs that have been subjected to butyric acid treatment. In both cases a membrane is produced around the egg, clearly visible with a low power of the microscope. In both cases this membrane, the vitelline membrane, often called the fertilization membrane,[1] becomes evident almost immediately and very much toughened on standing. The fertilized egg cleaves, gastrulates and later swims: the same is also true of the butyric-acid-treated eggs if they are exposed to hypertonic sea water at the proper time. The end results in the two cases are indistinguishable. Is there then also a physiological parallelism between the two kinds of activation?

It has long been known that an egg once activated by a spermatozoön does not respond to a second sperm insemination. Is this also true of eggs activated by butyric acid? If so when does the egg acquire such a physical or physiological state that it will not respond to the influence of a spermatozoön?

[1] For details of membrane production see Heilbrunn '15.

A great number of experiments have been performed in an attempt to find out the conditions under which fertilization may or may not be superposed upon butyric acid parthenogenesis and to define the boundary line limiting the effect of the spermatozoön following parthenogenetic treatment.

In production of parthenogenesis by butyric acid, eggs are exposed to an optimum concentration of acid in sea water and transferred to sea water at given intervals of time. Membranes do not form in the acid, but only after transfer to sea-water, and they are formed best and in highest percentage after an optimum exposure. 50 c.c. sea-water + 2.8 c.c. $n/10$ butyric acid was found to be a good concentration, and the optimum exposure in this concentration was usually about 20 seconds. After either too long or too short an exposure the percentage of membranes was less and they were less well formed.

The general method of conducting the experiment is as follows: Eggs are collected, washed three times, and divided into equal lots; the lots are subjected separately to butyric acid using the entire 52.8 c.c. for a graded series of time intervals (see Table I.).

TABLE I.

No.	Length of Acid Exposure.	Percentage of Membranes.	Percentage of Cleavages (2 Cells or More).
1	3 sec.	0	96
2	6 "	20	70
3	10 "	50	35
4	15 "	85	3
5	20 "	95	3
6	25 "	70	4
7	30 "	40	8
8	45 "	1	26
9	1½ min.	0	35
10	2 min.	0	45
11	3 "	0	30
12	4 "	0	30
13	5 "	0	25
14	7 "	0	25
15	9 "	0	4
16	11 "	0	0

For each lot the acid solution previously thoroughly mixed was poured over the eggs and at the given time eggs and butyric acid solution were poured into vessel A, containing 1 liter of sea-water + 3 c.c. $n/10$ NaOH, to stop the action of the butyric

acid; membranes were counted by using the Leitz 7 mm. objective and ocular 3. Eggs were then transferred from vessel *A* to vessel *B* containing 100 c.c. fresh sea water, carrying over from 2 c.c. to 5 c.c. of the water from *A*. Immediately there was added to *B*, 4 c.c. of a sperm suspension (2 drops dry sperm + 100 c.c. sea water) freshly made up for the experiment. Cleavages were counted about the 8 to 16-celled stage. The approximate time of an entire experiment is one and one half hours, but the time factor is neglible both as regards the length of time the stock supply of eggs has remained standing and in regard to possible deterioration of the sperm suspension.

The following experiment was adapted to ascertain at what stage, if any, in the action of the butyric acid, capacity for fertilization was lost and to relate such results to the parthenogenetic effect. The results during two summers have been entirely consistent.

Experiment 29, July 23, 1915.

Eggs after washing were divided into 16 lots. Each lot was given a certain exposure to butyric acid (50 c.c. sea water + 2.8 c c. *n*/10 butyric acid), the action of acid checked by transferring to alkali sea water, and eggs inseminated with sperm in a dish of fresh sea water.

These results are tabulated in a curve, Fig. 1 (*a* and *b*). The axis of the ordinates represents the percentage of cleavages and the axis of the abscissæ the length of exposure to butyric acid in seconds.

In a first glance at such a table and curve four points are very strikingly revealed to us: (1) The percentage of fertilization decreases as the number of membranes increase. (2) After membranes cease to appear there is a gradual rise in the percentage of cleavages: (3) From a longer exposure to butyric acid cleavages again fall off until (4) after ten minutes' exposure to butyric acid (varying in individual experiments) the fertilization reaction is no longer induced by sperm. Let us look then at the process of membrane production a little more closely.

The concentration of the butyric acid in sea water used caused no visible change around the cortex of the egg following a

three second exposure, with the exception that usually all of the transparent jelly layer around the egg was completely dissolved: no membranes were produced and the eggs appeared entirely normal. After six seconds' exposure, however, usually a small per cent. of the eggs showed a distinct membrane, ordinarily quite small, while others had begun to show only very slight indications of a membrane. Membrane production increased in intensity and in number with increase in length of exposure until practically all eggs had produced membranes indistinguishable from membranes produced at fertilization. In this experiment 95 per cent. of the eggs had produced membranes following an acid exposure of 20 seconds.[1] Longer acid exposure gave a gradually declining curve of membrane production usually falling off so rapidly that eggs exposed to the same strength butyric acid for 1 minute produced very small membranes or none at all until finally almost no indication of any cortical change was evident. There is decidedly a quantitative aspect to membrane production shown in these experiments, and it is to what shall be called the optimum condition of membrane production that we will direct our attention a little later in this paper.

As above mentioned the fertilization capacity (indicated by cleavage and development) falls off gradually as the percentage of membrane production increases. When the optimum conditions for membrane production are given, fertilization is restricted to a very few eggs or is entirely absent. In very few experiments does one obtain 100 per cent. of membranes by butyric acid treatment and usually the percentage of cleaving eggs following insemination, corresponds very closely to the number that did not produce membranes. After an acid exposure of the given concentration, lasting only three to five seconds, insemination causes practically all eggs to produce characteristic membranes; cleavage is essentially normal and a very high percentage of swimming larvæ are obtained. With longer exposures (10, 15, 20 sec.) the number of eggs that cleave after insemination is less than with the shorter exposure. This decrease in number of cleavages continues as the length of exposure increases up to one minute or longer when there is a

[1] The optimum time varies slightly under different sets of conditions.

gradual increase in the number of cleavages. But development after this length of butyric acid exposure is not preceded by membrane production.[1] From an exposure of one minute the percentage of cleavages continues to rise as the length of exposure is increased, until the greatest number of cleavages is obtained after an acid exposure of approximately two minutes (varying with different lots of eggs). From this length of exposure to longer ones the possibility of fertilization gradually decreases until exposures from five to ten minutes to the given strength of butyric acid absolutely prevents fertilization of the eggs.

It is of significance that the majority of eggs fertilized after a prolonged butyric acid treatment cleave very irregularly when cleavage is present at all. Cleavage many times is delayed, cells appear very unequal in mass, the usual radial type of normal cleavage is lost and the pattern becomes very obscure; nuclear division may appear when no evidence of cytoplasmic cleavage is present.

Likewise the larvæ that result from eggs over exposed to butyric acid are very abnormal. Usually they are slightly retarded in their development as compared with a similar stage of differentiation in a normal series. The degree of abnormality in such lots is extremely variable. Many of the eggs do not cleave at all; some go to 2, 4, 8, 16 cells or farther and disintegrate. Most of the larvæ do not swim at the surface of the water but remain on or near the bottom of the dish. Almost any form from a rounded mass of protoplasm barely exhibiting a slight quivering or very slow spinning movements up to normal individuals may be observed. Forms having only one appendage representing an arm or others with extremely long arms very closely bound together, arms widely separated—even to 180°—large oral lobes, scarcely visible oral lobes, or very irregular masses of protoplasm with blunt projections, were noted. The rate of mortality was usually very high in these abnormal lots. With an exposure approximating the point at which no fertilization is possible, no normal larvæ were obtained; irregular masses of protoplasm scarcely resembling a normal larva were the only

[1] Herbst, '06, has called attention to the fact that eggs over-exposed to butyric acid do not produce membranes when inseminated with sperm.

forms noticed that gave any evidence of life at all; and none of these reached the pluteus stage of development.

Fertilization is the normal reaction between a ripe male and female sex cell of the same species. Eggs may be led on to development by artificial means, chemical or physical, but the writer considers it just as improper to speak of fertilization in these cases as to call eggs that have produced membranes only, as a result of certain substances extracted from sperm, fertilized eggs (Robertson '12). *Penetration of an egg by a spermatozoön is not fertilization.* Certain internal conditions of each of the partners is necessary for the fertilization reaction and sperm may enter eggs when these conditions are not right for the reaction; but in an instance of this kind we do not have fertilization. In the conditions observed above we obtain a certain amount of development even though it deviates widely from the normal conditions of this process. Some of the eggs start the cleavage process but do not complete it; others go farther only to fail in the attempt. This development results from some reaction between egg and sperm since uninseminated eggs under the same conditions do not cleave. The conditions for the normal reaction have only been partially fulfilled and these eggs have been fertilized only incompletely. We may speak of such conditions as partial fertilization and thus recognize the quantitative aspects of fertilization as we do the quantitative aspects of artificial parthenogenesis.

But why, may we ask, do eggs that have produced membranes as a result of butyric acid give no evidence of fertilization whatever, even though they have been heavily inseminated with sperm? Is the membrane the only barrier to fertilization? If a sperm should come in contact with the egg cytoplasm or even enter it could it fertilize the egg?

Activation of the egg by the optimum exposure to butyric acid results in membrane production: so also does activation by sperm produce membranes. In neither case is subsequent insemination by sperm effective. Wilson ('03) has shown for *Cerebratulus* that pieces cut from fertilized eggs and dropped mmediately into water containing sperm do not develop even though pieces of much smaller size cut from unfertilized eggs do

develop. The cytoplasm is incapable of responding to the influence of the sperm. Practically the same thing has been shown for the starfish egg by the studies of Delage on merogony.

Can the failure of the butyric treated eggs to respond to the presence of sperm be explained in the same manner and if so what is the physiological condition of the egg that causes its behavior? Obviously we shall have to examine very closely the condition of the egg at the close of the activation process induced by butyric acid treatment.

A. Optimum Exposure to Butyric Acid.—We will turn our attention to the point in the fertilization curve (Fig. 1, 20 seconds) at which we obtain membranes surrounding the eggs that are indistinguishable from those produced as a result of fertilization. The membranes are comparatively of the same size as those produced at fertilization; the hyaline layer appears shortly after the acid treatment and so far as one is able to judge, the membranes in the two instances are morphologically identical. The production of membranes in both cases has been effected by the egg; certain processes have been set in motion by two different activating agents and in each case we have obtained distinct membranes. Development proceeds in the case of fertilization, but in artificial parthenogenesis usually it does not go farther unless the eggs are subsequently treated with hypertonic sea water. Many agents may be utilized in causing eggs to produce membranes but not any of them allow the egg to reach the stage of swimming larvæ unless the treatment is followed by hypertonic sea water or other secondary treatment. Evidently the only common factor in the two series of events is the egg itself. It is inconceivable that development could proceed after such a variety of agents as may be used without passing through at some time some of the same conditions as does the normally fertilized egg.

Immediately or within a short time after membrane production in fertilization (as we have mentioned) changes in the physiological state of the cytoplasm have rendered it incapable of further response to a spermatozoön. Does the same condition exist in case of membrane production by butyric acid or is the absence of fertilization at this point merely due to the presence of the membrane as Loeb has suggested.

Quoting from Loeb ('13, p. 234):

"If we call forth artificial membrane formation first by butyric acid, no spermatozoön can enter the egg, since the fertilization membrane is impermeable to a spermatozoön. But we can destroy the membrane by shaking it. If we then add sperm to such eggs, the spermatozoa enter, cause a second membrane formation (in which the membrane fits tightly around the egg)[1] and the eggs develop at room temperature without requiring any further treatment with the hypertonic solution; . . ."

(*a*) *Membrane Production.*—If the facts are as Loeb states them we can only come to the decision that no change in the physiological state of the cytoplasm has occurred; at least it has not rendered the cytoplasm unfertilizable. If we could eliminate the membranes from all the eggs we should be able to obtain practically as high a percentage of developing eggs as before the butyric acid treatment. Furthermore if the spermatozoön carries a lysin-like substance that causes membrane formation there is no reason that we should not get a second membrane formation around the egg following insemination.

The first point for consideration is the character or degree of membrane production. This is essential. We have already seen that there is a decided quantitative aspect to the process and that the power of fertilization runs parallel with this.

Quoting further from Loeb ('15, p. 262):

"The treatment of the eggs of *Arbacia* with butyric acid leads to the formation of a membrane which varies considerably in the eggs of the same female. . . . Since the membrane called forth by butyric acid is not always plainly visible, it is a prerequisite that always one set of such eggs should be set aside as controls to ascertain whether or not all the eggs disintegrate rapidly (if no second treatment is given to them). Only if they all disintegrate rapidly have we any guarantee that in all of them the membrane formation has been effective."

Loeb however fails to consider that disintegration is by no means a test for membrane production. Eggs exposed to butyric acid for slightly longer than the optimum time for membrane production do not produce membranes yet they

[1] Loeb, '15, has later retracted this second membrane formation.

cytolize even more rapidly than in cases where membranes have been produced. Practically all pigment diffuses from eggs so treated, when allowed to stand, within a very few hours; they will, many times, have almost completely disintegrated when the first signs of cytolysis appear in eggs provided with membranes. Furthermore, what evidence of membrane production have we when the membrane is not visible? We have no reason to doubt that partial fertilization, at least, is possible in instances where no membranes appear following the acid treatment; membrane production has not been initiated. Activation has, at best, been only partially effected; the reactions may have been started but they were not completed. It behooves us to look a little more closely at the process of the butyric acid treatment.

The best method for causing membrane production in the egg of *Arbacia* is the famous butyric-acid method developed by Loeb. But, as all who have used it know, great care must be exercised, to obtain the best results. As Loeb has pointed out different lots of eggs respond very differently to essentially the same treatment. But if these facts are borne in mind very good results may be obtained. The eggs used in my experiments have always been washed from two to four times before treatment. Unwashed eggs do not usually give good results; many eggs do not produce membranes. One variable feature in obtaining good membranes is the length of time the eggs are allowed to remain in the acid. This is probably dependent somewhat upon temperature as well as upon the conditions of the individual eggs. In some of my experiments I have obtained the best results (highest percentage of eggs producing membranes and membranes appearing normal in size) from exposures lasting only fifteen seconds; at other times longer exposures give better results. This sometimes is obtained at twenty-five to thirty seconds but usually it is near twenty seconds that the better results are obtained. Acidity is a factor that must be considered: a very slight amount of acid after the transfer prevents the reaction from being complete. Thus I have found in some cases that a solution into which the eggs have been removed following the acid treatment, that is as slightly acid as $n/1{,}600$ would entirely prevent membrane production.

Large quantities of eggs should not be treated with a small amount of acid solution if good results are desired. In all my experiments the same dilution of butyric acid has been used (50 c.c. sea water + 2.8 c.c. $n/10$ butyric acid) the entire 52 c.c. has been employed and seldom has a larger quantity than 2 c.c. of eggs been treated at the same tme. In order to avoid the excessively large quantity of sea water necessary to dilute this acid to a point where it would no longer inhibit membrane production, a known amount of $n/10$ NaOH has been added to one liter of sea water into which the eggs and acid were poured: a sufficient quantity was used that after the eggs and acid were added the solution was still barely alkaline to phenolphthalein, and finally eggs were observed under the microscope and the percentage of eggs possessing membranes was noted. With these points in mind we may turn our attention to the possibilities of fertilization following such an optimum treatment with butyric acid.

(*b*) *The Effect of Sperm, after Elimination of Membrane.*—The method used is that employed by Loeb on studies of this nature, *i. e.*, production of membranes by butyric acid, shaking the eggs in a test tube to remove the membranes and subsequently inseminating with sperm. Loeb was sometimes doubtful as to the efficiency of shaking in removing the membranes, some were only torn according to his account and where a small percentage of cleavages was obtained it was very convenient to consider that these holes had again closed over and prevented entrance of the sperm. If the shaking process is conducted quickly enough after the production of membranes they are very easily removed. Thus in one lot of eggs 90 per cent. of which revealed typical membranes, shaking[1] was conducted 3 minutes after being returned to alkaline sea water. Examination by means of the microscope did not reveal the presence of a single membrane out of 100 eggs counted. Six minutes after membrane production, as nearly as possible the same amount of shaking was given to another batch from the same lot, yet 30 per cent. of these eggs still possessed intact membranes; nine minutes after, even

[1] Shaking was conducted in a test tube 3 × 21 cm. containing eggs and sea water. The tube was filled to one third its volume.

though the eggs were given as nearly as possible an exact equivalent shaking as the first, yet 55 per cent. of them possessed intact membranes. The membrane becomes very much toughened shortly after production and a much greater amount of mechanical agitation is necessary to destroy it.[1]

Even though many eggs are broken to pieces during the shaking process and the solution becomes very highly colored from escaping pigment, yet the eggs remaining intact appear in good condition, not different in appearance from normal unfertilized eggs. The shaking has not materially affected their developmental capacities; when such eggs are subjected to a hypertonic treatment one can obtain from 35 per cent. to 40 per cent. of swimming larvæ.[2]

Observations are entirely limited to experiments in which eggs possessed large full membranes, and to lots of eggs that showed a high percentage of membrane production. All other sets of eggs in which membrane production was not of the best were discarded. At variable times of the season short periods appear during which it is very difficult to obtain a large per cent. of good membranes and usually the percentage of development from normal insemination is very low. At such times no experiments of this nature were performed, but for all other periods in which observations have been made throughout two summers the results have been entirely consistent.

The following experiment, a typical one, will serve to present the method of treatment and the results obtained from this line of study.

Experiment 23 B. July 19, 1915.

3:00 P.M. Eggs collected and washed.

5:00 P.M. Butyric acid exposure (50 c.c. sea water + 2.8 c.c. *n*/10 butyric acid).

[1] That the membranes have been completely shaken off the egg and have not simply collapsed is indicated by the fact that eggs which have been shaken less vigorously than others, still possess pieces of membrane adhering to the surface of the egg; also by the fact that immediately after shaking, no indication of a membrane is present, and that shortly a new surface film, the hyaline layer, appears around the egg.

[2] The number of cleavages is considerably greater. In a great many eggs the blastomeres fall apart, due to lack of a membrane, and do not reach a swimming stage.

5:00:20 P.M. Eggs and acid water poured into 1 liter of alkaline sea water.

(*a*) 97 per cent. of eggs possessed good membranes.

5:02:20 P.M. One part of (*a*) eggs shaken in test tube 12 times, poured into normal sea water.

(*b*) 10 per cent. of eggs still possessed full or partial membranes.

5:05 P.M. (1) (*b*) eggs in 100 c.c. fresh sea water + 4 c.c. sperm suspension.

5:20 P.M. (2) (*b*) eggs in 100 c.c. fresh sea water + 4 c.c. sperm suspension.

5:23 P.M. (3) (*a*) eggs in 100 c.c. fresh sea water + 4 c.c. sperm suspension.

5:40 P.M. (4) (*b*) eggs in 100 c.c. fresh sea water + 4 c.c. sperm suspension.

5:45 P.M. (5) normal insemination—control.

After 3 hours the number of cleavages were noted and after 24 hours observations were made for swimming larvæ.

Cleavage Out of 100 Eggs Counted.	Swimming Larvae.
1..... 0	0
2..... 0	0
3..... 1	0
4..... 0	0
5.....95	85 estimated.

Thus even though we have entirely deprived 90 per cent. of the eggs of their membranes yet in no case did we have a single egg showing cleavage after insemination. One per cent. of cleavages were noted for the unshaken control and we see that 97 per cent. of the eggs formed full membranes. Cortical changes though slight were appearing on the other eggs.

The shaken eggs, (*b*) lot, cytolized even more rapidly than did the unshaken (*a*) lot. This lot (*a*) still possesses its membranes.

Our conclusions from this, as well as from many other experiments, can only be that these eggs, after production of full membranes, are in an unfertilizable condition. This condition is not due to the presence of a so-called impermeable membrane for that we have eliminated by shaking; neither is it due to injury of the eggs through shaking for a large percentage of

swimming forms are obtained after a short hypertonic treatment. We can not account for it on the basis of a weak sperm suspension because normal eggs with the same insemination gave 95 per cent. of cleavages. Many times too but with no better results an excessively heavy insemination has been used. Standing for a short time before insemination has no different effect; indeed sperm retain their motility and actively swim around in the dish several hours after insemination. The eggs do not again return to the fertilizable condition by standing in normal sea water at room temperature. Cytolysis is more rapid than where membranes are retained and in the course of twelve hours most of the eggs have gone to pieces.

Neither has the percentage of fertilization ever been able to be increased by the use of varying amounts of NaOH.[1] In experiments in which a few of the eggs did not produce membranes as a result of the acid treatment, controls often show a corresponding number of fertilizations. *But in no case were fertilizations increased by eliminating the membranes produced after acid treatment.* Even though membranes may possibly exert some influence in keeping out accessory spermatozoa yet this is not the essential block to fertilization.

Obviously the eggs are not fertilized upon addition of sperm. Can it be possible that yet some physical barrier exists at the surface of the egg protoplasm?

Cytological observation of such experiments have shown an exceedingly interesting and significant situation. The observation will be presented in a very general way in the following section.

(*c*) *Cytological Results of Optimum Butyric Treated Eggs.*— Before presenting the data from a cytological study it may be well to dwell a little on the significance of such a study in relation to the partially antagonistic hypotheses, the lysin theory of fertilization and the fertilizin theory.

From the essential conceptions of the lysin theory obviously we should expect no aberrant behavior either on the part of

[1] Loeb has shown that under certain conditions variable percents of NaOH added to inseminated dishes materially aids in increasing the percentage of fertilizations.

sperm or egg in their union and further behavior following insemination, after artificially induced membrane production. The fundamental limiting factors are bound up in cortical, physical phenomena and these being entirely removed, the normal processes should go on unmodified.

We should naturally expect in instances of this kind: (1) Reactivation of the egg; (2) penetration of the spermatozoön; (3) normal development.

(1) has already been considered and proven to the contrary. Sperm are active, come in contact with eggs that have been entirely deprived of their membranes yet they never prove themselves capable of inducing the formation of a new membrane. The lysin-like substance fails to act. (2) will be considered from a study of sections while (3), like (1), has been proven negatively. These eggs do not develop in response to sperm but disintegrate as readily, or more so, than do eggs possessing membranes. If sperm should enter these eggs, and there is nothing in the conception of this theory to indicate that it is not so, the spermatozoön should exhibit its corrective effect and allow the eggs to develop normally. If then sperm are found within these eggs which give no external indications of development every postulate of the theory will have been disproved and consequently it will have to be discarded.

The facts already presented however find a ready interpretation in terms of the fertilizin hypothesis. Since activation has already been once accomplished, through butyric acid, further influence toward development by means of sperm is entirely negative. From the postulates of this theory we should not expect (1) reactivation; we may or may not expect (2) penetration of spermatozoa and we do not expect (3) further development after insemination.

(1) and (3) need not here be further discussed; in reference to (2), penetration of a spermatozoön is not the point of greatest importance but rather the question of a reaction between egg and sperm. We do not know all the factors involved in penetration. Whether or not the spermatozoön is carried into the eggs in a purely passive state or is itself actively engaged in the process will not be discussed at this point. Of greater significance

at this time is the question of the possibilities of an influence exerted by the sperm, provided it could enter the egg by any means whatever after activation by butyric acid. We should, from hypothesis, expect the reaction to be entirely absent and this I shall prove is the fact. In these preserved series the experiments were conducted in the same manner as that given on page 152. After membranes had been produced as a result of butyric acid and destroyed by shaking, the eggs were transferred to fresh sea water and inseminated very heavily with fresh sperm. Eggs were preserved at definite periods by killing in Boveri's picro-acetic acid, Bouin's mixture, and Meves' fluid. Sections were cut in paraffin at 4 μ and stained with a fresh solution of iron-hæmatoxylin. The better results were obtained by staining, following Boveri's picro-acetic acid, in a 0.5 per cent. aqueous solution of hæmatoxylin first dissolved in a small amount of absolute alcohol and diluted properly with distilled water, using the stain immediately. This gives a clear gray cytoplasm, against which the intensely black sperm heads stand in marked contrast.

Eggs preserved fifteen minutes after insemination reveal the fact that sperm have entered in large numbers; also that entrance of one sperm does not prohibit the entrance of others. A section of an egg 4 μ thick may show any number of sperm heads from one to twenty or sometimes more. These spermatozoa are scattered all through the cytoplasm and are not located near one side where accidental injury to the egg might have made entrance possible; they are found at any level of the egg. The majority are located near the periphery of the egg but many are found at the center, close to the egg nucleus and even in loose contact with it. The periphery of many eggs is literally packed with the sperm heads. There seems no definite orientation; sperm side by side or in groups may be turned in opposite directions or yet may lie at right angles or in practically any direction to each other. Evidence of active behavior of such sperm is entirely negative. No asters arise around the sperm heads and neither do they exhibit the tendency to become enlarged and vacuolated as in normal fertilization, especially as the male and female pronuclei approach each other. The only discernible

difference between sperm within the cytoplasm and those remaining outside the egg but in contact with the surface is the absence of a tail. No tail has ever been observed within the cytoplasm of these eggs.

Thirty minutes after insemination we see no evidence of any change on the part of the sperm within the egg. The egg nucleus however has begun to change somewhat. The nuclear wall has disappeared in some and chromosomes are definitely formed usually lying scattered, or more aggregated in the cytoplasm. Slight radiations have begun to appear centering indistinctly near the nucleus or chromosomes. In a few eggs, the percentage increasing at a later stage, chromosomes are dispersed along the rays that diverge from the center of the egg. Practically no amphiasters have ever been found in these series.[1]

During all this change on the part of the egg pronucleus the sperm heads have given not the slightest indication of any change. They lie in the cytoplasm very much as if they were foreign bodies.

From Hindle's cytological study of artificial parthenogenesis in *Stronglyocentrotus purpuratus*[2] we know that essentially the same nuclear changes are present after butyric acid treatment alone that we have in the above lot of eggs. Monasters are formed but not amphiasters. And in the absence of any indication of a reaction due to the presence of sperm we necessarily must conclude that these changes have been induced as a result of the butyric acid treatment and not by the effect of sperm. There has been no fertilization; the character of the egg has become changed to such an extent that no activation whatever so far as one may judge from experiments and sections, has been effected by the spermatozoön. The ovogenous substance has once been activated (*i. e.*, by butyric acid) and further activation by sperm is just as impossible as it is after normal fertilization.

B. Over-Exposure to Butyric Acid—Partial Fertilization.—Again referring to the curve of fertilization we notice that exposure to butyric acid slightly above the optimum does not

[1] In one series of preserved eggs, two containing amphiasters were found; but the control of the experiment—eggs inseminated without the membranes being destroyed—showed 6 per cent. of cleavages.

[2] Archiv. für entw' Mech., Bd. 31.

result in the production of membranes; we have also noted that insemination is followed by a certain amount of abnormal cleavage and a corresponding percentage of aberrant larvæ. Such conditions were spoken of as partial fertilization. We see many evidences in the literature of such quantitative aspects of both artificial parthenogenesis and fertilization. These conditions may be brought about either by treatment essentially effecting the normal condition of either parent cell before union—an internal modification—or by allowing union of the two elements in an environment in which the normal processes involved in union are modified—an external modification. No attempt to review the very many conditions and instances of the production of such abnormal conditions will be made. It is sufficient to point out the fact that very gross abnormalities have been produced by modifying the processes of fertilization. One of the best demonstrations of the quantitative effect of artificial agents in starting off the dormant mechanism of the egg is that given by R. S. Lillie ('15).

Lillie found that a brief exposure of unfertilized eggs of *Asterias forbesii* to temperatures ranging from 32° C. to 38° C. caused membranes to be produced. If these eggs were then treated with hypertonic sea water development ensued and larvæ were produced in large numbers. Further than this the effect of the hypertonic solution could be replaced by a second exposure to high temperatures or by butyric acid. Either was capable of partial imitation that could be completed by a further treatment of the same or other agent. In the sea urchin however the conditions seem to be more specialized. Optimum conditions for exposure to butyric acid results in the production of membranes and at this time fertilization is impossible. But a longer exposure does not result in membrane production and in this condition a certain amount of reaction between egg and sperm is evident from the fact that cleavage and larvæ are produced.

The physiological differences in the two cases will be partially dealt with in Sec. V.

(*a*) *Cytological Observations.*—Cytological preparation of these instances of partial fertilization are extremely interesting, and vary in essentials but little from those previously described by

the Hertwigs ('87). Sperm enter these eggs over-exposed to butyric acid, in large numbers and evidently at any point on the circumference. Eggs exposed to butyric acid for $2\frac{1}{2}$ minutes were inseminated, preserved and sectioned. Fifteen minutes after insemination the eggs contained one or many sperm lying in the cytoplasm either unchanged or in various stages of activity. Many possessed asters and some even though quite widely removed from the egg nucleus, appear very much like the swollen sperm in normal fertilization just before copulation of the nuclei. Many sperm were undergoing fragmentation with no indication of an aster.

Forty-five minutes after insemination many eggs had formed amphiasters appearing quite normal. Polyspermy however seemed predominant: many figures were composed of three, four, or five spindles variously linked together; different sets of amphiasters within the same egg but completely isolated from each other are often to be noted. Large numbers of sperm may be found bunched together in a mass, the whole being surrounded by protoplasmic radiations that extend only a short way through the cytoplasm. Such sperm masses usually give evidence of quite an extensive amount of disintegration. Many of the eggs with definitely formed chromosomes dispersed along the rays of a monaster show sperm lying inactive. Many eggs also are present in which neither egg nor sperm seem to undergo any change, at least, wi hin the space of two hours.

In a series of this kind we see all gradations of completeness of the process of fertilization from total absence of any reaction to processes very greatly resembling the normal behavior of the two elements. Some of the eggs have evidently suffered more from the activating influence of butyric acid than others and are consequently less responsive to the normal stimulus exerted by a spermatozoön.

C. Prolonged Exposure to Butyric Acid.—The last point in the fertilization curve to be considered is the point at which fertilization absolutely ceases. From the highest point in the curve after an over-exposure to butyric acid, a gradually declining amount of fertilization is possible until no indications of a reaction are at all visible. This is from five to fifteen minutes'

exposure to the acid varying with conditions. Eggs over-exposed to the acid agglutinate heavily, but they neither produce membranes nor disintegrate exceptionally fast. The individual eggs vary in appearance but little from normal unfertilized eggs but all efforts to fertilize such eggs have resulted in complete failure.

Why has the fertilization capacity disappeared? If sperm could enter these eggs would the reaction appear?

(*a*) *Cytological Observations.*—From the experiments, lots of eggs were preserved that had suffered the shortest possible exposure that would prohibit cleavage.

Examination of sections revealed the fact that practically all the eggs contain sperm nuclei, many sections showing 8 or 10 in one plane of section. They are located at all planes of the egg from periphery to center. These sperm however give no evidence whatever of being either effective or effected. Similar to the conditions encountered in the optimum butyric acid exposure the sperm do not produce asters. They appear entirely as foreign bodies suspended in the cytoplasm. Their contour has suffered no noticeable change but they retain their usually oblong pointed appearance. They do not react with the egg. Something is absent with which or to which they normally react.

We can not conclude that the eggs are dead and thus veil our ignorance, for how then could we account for the presence of spermatozoa? It is inconceivable that the spermatozoön could acquire enough momentum to penetrate the surface of the egg and to carry itself on through a mass of protoplasm very roughly estimated as 15 to 20 times its own diameter. It cannot bore its way through, for were it possessed of a perforatorium (which it is not) the tail would be completely buried long before it reached the center of the protoplasmic mass even if the tail remained in contact with the sperm head. The very fact that spermatozoa have entered the eggs is proof that the eggs are not dead. The normal environment for the activity of the sperm has been changed. Something again is absent that would normally permit the reaction and the consummation of development.

IV. Effects of a Rise of Temperature on Fertilization.

While studying the effects of altered temperatures upon the process of development the writer found that by subjecting sea

urchin eggs to sea water, the temperature of which had been raised to 35° C.–40° C. fertilization could be entirely prohibited yet the eggs remained apparently normal and showed no signs of cytolysis.[1] No attempt to restore the fertilizable capacity was successful.

Numerous instances are to be found in literature relative to the powers of a rise in temperature to initiate development.

Delage, '01, determined that starfish eggs developed parthenogenetically if immersed in sea water of 30° C.–35° C. at the time of rupture of the germinal vesicle.

R. S. Lillie ('15) found that exposing maturing starfish eggs to a temperature of 29° C.–36° C. for a short time produced no visible changes in the egg; slightly longer exposures however led to the production of typical membranes; but with scarcely no further development the eggs break down before and after cleavage. But an exposure three or four times as long as one necessary to give rise to membranes, produces favorable development and a large number of swimming larvæ are obtained.

Miss Allyn ('12) found a rise in temperature very effective in producing cleavage and development in the egg of *Chætopterus*. Temperatures of 32.5° C. and 34.5° C. proved the more favorable for cleavage and development of this egg: a small per cent. of swimming forms were obtained. Temperatures of 35° C. and above are less effective: no swimmers were obtained and cleavage was very abnormal.

The Hertwigs ('87) by subjecting the eggs of *Stronglyocentrotus lividus* to abnormal temperatures determined that fertilization after a certain length of exposure was prohibited. An exposure of 5 minutes at 35° C.–36° C. gave essentially the same results as a ten-minute exposure to a temperature of 31° C. They noted and emphasized the progressive effect of the higher exposures, revealing again the quantitative aspect of whatever changes are effected within the egg. In using a constant time—five minutes—the effect of a temperature of 31° C. was scarcely noticeable, the process of fertilization was essentially normal: at 35° C.–36° C. however changes were noticeable. The eggs

[1] Von Knaffi, '08, found that heating sea urchin eggs to 41° C. caused cytolysis in a very short time.

usually produced a more or less typical membrane but later cleavage was very abnormal. In many of the eggs there could be seen more than one spermatozoön.[1] Following an exposure to 39° C. and subsequent insemination, very few of the eggs produced membranes, and cleavage if present at all, was very irregular. After an exposure to 41° C.–42° C. and 45° C.–47° C. no development was obtained by the addition of sperm.

It is very evident that different eggs react to the heat stimulus very differently. The eggs of *Chætopterus* and starfish seem to possess much less stability than do eggs of the sea urchin. They are much more easily started out in their development. Thus a slight rise in temperature is very effective in producing cleavage in the two former but so far as the writer is aware almost entirely negative results have been obtained with the urchin egg. In common with the more responsive egg however is the fact that exposure to high temperatures causes a loss in the capacity for fertilization. It was in a study of the character of the change effected in the egg that these few observations have been made. The writer was not familiar with the experiments of the Hertwigs with heat, at the time of his observations. The experiments on the egg of *Arbacia* agree essentially with their earlier observations in a study of the egg of *Stronglyocentrotus lividus*.

The experiments were conducted by transferring washed *Arbacia* eggs into sea water, in a small beaker, the temperature of which had previously been raised to that desired for the experiment. The beaker was partially immersed in a water bath of the correct temperature and a thermometer recorded the temperature of the two vessels. Eggs were removed from the beaker of warm sea water at certain time intervals, transferred to finger-bowls of normal sea water and inseminated with a fresh sperm suspension.

Many experiments were conducted using various temperatures from 25° C. to 45° C. but only the results of a constant temperature will be reported at this time.

Experiment.—Unfertilized eggs of *Arbacia* were exposed to sea water of a temperature of 35° C., removed to normal sea water and inseminated. The results are tabulated in Table II.

[1] Revealed by staining eggs whole.

TABLE II.

No.	Time of Exposure.	Percentage of Cleavages.	Percentage of Swimming Larvæ.
1	3 min.	85	70
2	5 "	50	35
3	7 "	3	0
4	9 "	2	0
5	10 "	0	0

It was to determine whether sperm entered eggs thus treated that a number of series were preserved and sectioned. An endeavor was made to obtain eggs just at the critical point (*e. g.*, at the shortest length) of exposure at which fertilization was prevented.

1. *Cytological Observations.*

Sections of such eggs reveal the fact that sperm have entered them in great numbers. Fifteen minutes after insemination sperm are found scattered diffusely at all levels throughout the egg, at the periphery and in the center, some lying very near the egg nucleus. So far as concerns any reaction characteristic of fertilization, it is entirely absent; no asters have ever been found in these eggs, as well as no indication of spindle formation or copulation of pronuclei.

The sperm heads have begun to lose their normal characteristic shape and have begun to disintegrate; some appear swollen with a tendency to become vacuolated, others appear to be falling apart in fragments. Many dark-staining granules are found through the cytoplasm evidently being derived from broken-down sperm.

The egg pronucleus has to a great extent lost its power of holding the stain and appears only a shadow of the original nucleus. It is still intact with little or no indication of destructive changes.

Thirty minutes later (45 minutes after insemination) eggs from the same lot present quite a different picture. The egg nucleus remains intact but does not appear stained. The spermatozoa appear to fall into two very general classes.

1. Those in which the nucleus has a tendency to disintegrate, scattering heavily staining granules throughout the cytoplasm.

2. Those that have a tendency to become vacuolated and to

increase enormously in size. The sperm head becomes broken up into pieces but these are retained at the periphery of a clear vesicle and are not scattered promiscuously about in the cytoplasm. All sizes of these vesicles are present from a mere loosely arranged condition of the content of the sperm head to vesicles larger than the female pronuncleus. These vacuoles appear everywhere within the cytoplasm and are usually lined by heavy black granules.

The point to be emphasized is this. Sperm enter these eggs in large numbers; they are not only found lying just within the surface of the protoplasm but are found all through the egg cytoplasm, peripheral and central, yet these sperm never give the slightest indication of changes characteristic of fertilization. No asters have ever been found, no spindles have ever been observed, though hundreds of cases have been closely studied. Neither does the egg pronucleus give any indication of response following entrance of the sperm. These sperm nuclei become enlarged to a very great extent, the sperm become vacuolated and disintegrate but the eggs are not fertilized; they never cleave normally and no larvæ appear in the cultures.

No great claims can be made that these eggs have suffered the initial changes in fertilization for in no case has the writer recorded or seen a typical membrane produced as a result of exposure to higher than normal temperatures. The eggs however remain intact and exhibit no power of fertilization even though they become literally loaded with sperm. Immediately we must face the question, why do they not fertilize? What is the character of the change in the makeup of the egg?

Loeb has gone so far as to say that development is impossible on account of death. "I found that by merely warming sea urchin eggs to 34° C. or 35° C. the formation of a typical fertilization membrane can often, but not always, be induced. If the eggs are then cooled quickly, no cytolysis follows. Such eggs are no longer capable of development, since a temperature of 34° C. kills them."[1] Loeb's only criterion of death is failure to develop after the addition of sperm. By such an argument one could prove that eggs following complete membrane pro-

[1] "Artificial Parthenogenesis and Fertilization," page 185.

duction induced by artificial means were also dead, but it is a well known fact that a short exposure to a hypertonic solution results in the production of swimming larvæ. Sperm enter the eggs after membrane production but yet do not cause development. Likewise they enter heated eggs and are also ineffective. Von Knaffi ('08) found that heating unfertilized sea urchin eggs to 41° C. led to practically instantaneous cytolysis. But heated to 35° C. for 10 minutes they do not cytolize but remain intact for a considerable length of time. These eggs then are not dead: the very fact that sperm enter them and are found at all levels bespeaks a living condition. These eggs then do not develop because they are dead but because some change has been induced that renders impossible the reaction between egg and sperm. We shall consider this change more thoroughly in Sec. V.

V. Fertilizin and Fertilization.

1. *Introduction and Discussion.*

In the preceding section we have caused to be produced certain conditions within the egg that have rendered it incapable of being fertilized, yet in no case can we conclude that the eggs are dead. The initial conditions however we can not doubt have been changed; the egg system has been modified until it is physiologically different from the normal egg.

Previously the results of analogous conditions have stopped with a morphological description of the condition and a guess at what is happening. The debut of the fertilizin hypothesis however allows us to go a step farther: it allows us not only to postulate certain conditions but also to test for the condition and prove or disprove the hypothesis.

As pointed out in the introduction the basis of this theory rests upon the presence of a substance secreted by the normal egg. This substance, fertilizin, is considered as playing the active rôle in fertilization. Activation of this substance is held to be the introduction to and the essential step to the start of the processes or reactions involved in the process. It was further pointed out that this agglutinating principle of the supernatant fluid was not present in experiments in which

unripe eggs were used or where fertilized eggs, or eggs possessing full membranes, were being used. In all these cases the egg was entirely unresponsive to the active principle of sperm and Lillie was led to believe that "this substance is necessary for fertilization." If then this substance is bound or neutralized when the egg is fertilized or has been activated by artificial means we should not expect to obtain any agglutination action from cases where the fertilization reaction was entirely absent; but where fertilizable eggs are present we should be able to detect the fertilizin.[1]

The writer has carried out a large number of experiments with eggs variously modified by agents, natural and artificial, to see if it were possible to establish a curve of fertilizin exactly parallel to that of the curve of fertilization. It was however realized very early that such was not possible due in large part to factors that enter in secondarily. Lillie ('14, page 545) has called attention to the fact that a destruction of part of the eggs in a given lot leads to the liberation of substances that act antagonistically toward fertilizin. From an experiment giving a very considerable agglutination reaction one obtains a complete absence of it if the tube containing the eggs is shaken slightly causing eggs to disintegrate. Some substance within the egg acts in such a way that it masks the presence of fertilizin. To this antagonistic substance Lillie has applied the name anti-fertilizin. Extracts or secretions containing this substance do not reveal their true content of fertilizin. Lillie's table (p. 546) shows the reaction of such a solution. A dilution of the supernatant fluid 1/320 gives as strong an agglutination as the same extract undiluted, but this is not true of a secretion from fresh eggs. The agglutination reaction is a function of the concentration of fertilizin in the normal case but not so when anti-fertilizin is present.

The supernatant fluid from eggs exposed to butyric acid for 2–5 minutes is usually quite colored due to broken down eggs and the escape of substances from within the egg. After the prolonged butyric acid treatment substances gradually escape from the egg due no doubt to the increased permeability of the

[1] We must not be confused by instances where for purely physical reasons sperm are barred from entrance to eggs. In all the above circumstances we have seen from cytological preparations that sperm actually enter these eggs in large numbers.

egg surface. This is well illustrated by microscopical examination of eggs allowed to stand after treatment as well as by their behavior upon the addition of sperm. The following experiment will be instructive.

Experiment.

8:45 A.M. eggs collected, washed, divided into lots *A*, *B*, *C*, *D*.
9:01 A.M. to 9:10 A.M. *A*. exposed to butyric acid for 1 minute.
B. " " " " " 2½ minutes.
C. " " " " " 5 "
D. control.

A, *B*, and *C* poured into 1 liter of alkaline sea water to stop action of the acid and all were transferred, 9:15 A.M., to normal sea water, and allowed to stand at room temperature. Samples were removed at stated times and inseminated. The results of these inseminations, made within six hours after exposure to butyric acid, are given in Table III.

TABLE III.

	Removed from Stock and Inseminated.	Percentage of Cleavages.	Percentage of Swimming Larvæ.
A^1.....	9:20 A.M.	20	10
B^1.....	" "	50	10
C^1.....	" "	40	20
D^1.....	" "	90	90
A^2.....	11:30 A.M.	20	5
B^2.....	" "	45	5
C^2.....	" "	40	5
D^2.....	" "	90	90
A^3.....	3:00 P.M.	0	0
B^3.....	" "	0	0
C^3.....	" "	10	0
D^3.....	" "	80	90

We can readily see that conditions are becoming changed gradually; the fertilization capacity is being gradually reduced and substances diffuse from the eggs as revealed by the microscope. This diffusion of substances from the egg interferes very markedly with the agglutination reaction but despite these conditions very significant results have been obtained.

In reporting these experiments the writer has adopted the terminology and method devised by Lillie and he needs only here to review again the terminology employed and to very briefly restate the method.[1]

[1] For a more detailed account of the methods see Lillie, '14, Study VI.

Fertilizin is obtained normally in sea water that has stood over a quantity of *Arbacia* eggs in a test tube. The substance is liberated from the egg and passes out into sea water until the latter becomes highly charged with the egg secretion.

Sperm suspensions to be used as indicators are made up from the stock of "dry sperm."[1] Usually a 1 per cent. suspension was used (1 drop dry sperm + 99 drops sea water) and is mounted on a slide beneath a raised cover slip on the stage of a microscope, and the supernatant fluid to be tested is blown into the suspension by means of a fine capillary pipette, connected with a flexible rubber tube held in the mouth, while the sperm are in focus under a low power of the microscope.

In conducting the series of washings, sea water was added to eggs in a graduated tube and the volume of eggs and sea water denoted by the numerator of the fraction; the denominator represents the volume of eggs and sea-water remaining in the tube after the supernatant fluid has been removed. Each time after the addition of sea water, the tube was slowly inverted six times to insure a thorough mixing of the eggs and sea-water.

Some investigators have offered certain objections to the current interpretations of the agglutination reaction as well as to the facts encountered. Thus Loeb persistently contends, even in the face of definite proof to the contrary, that fertilizin is not a secretion of the egg but is only found in the clear jelly layer surrounding the egg. The present results however entirely confirm Lillie's contentions that eggs totally deprived of this clear jelly layer continue to liberate fertilizin in very great quantities. This jelly layer almost entirely disappears from the surface of the egg after a three-second exposure to butyric acid and it is entirely gone after five seconds; yet eggs exposed to the acid for two to five minutes or longer, and thoroughly washed by several changes of water, still continue to produce the agglutinating substance. Neither does it take a "fertilizin partisan," as Loeb has suggested, to see it. The writer has demonstrated the reaction to numbers of investigators at the Marine Biological Laboratory who had never before observed the longer, more intense, reaction of a secretion from untreated eggs. Indeed it

[1] See page 141.

is very difficult to obtain a lot of eggs following fertilization or membrane production that do not give a slight trace of fertilizin. Seldom has the writer obtained as high as 100 per cent. of normally fertilized eggs or eggs possessing butyric acid membranes, and unless this is realized there will still be eggs present continuously secreting this substance; and since a very few eggs produce enough to be readily detected one may be led into an erroneous interpretation of results. One must know the condition of the eggs with which he is dealing. We may repeat again—*the difficulty lies not in being able to obtain the reaction but in producing a condition in mature eggs in which fertilizin is entirely absent.*

2. *Conditions Where Eggs May Be Partially Fertilized.*

The following experiment is very instructive and gives the essential conditions of eggs at a few different points in the curve of fertilization. Many such experiments have been performed, each somewhat varied in the lengths of butyric acid exposures yet the general results have always been the same.

July 22. Eggs collected and washed, and divided into seven lots.

A. Control.

B. Butyric acid exposure 3 seconds—all jelly gone.

C. Inseminated. 5 minutes later, short butyric acid exposure to dissolve jelly.

D. Butyric acid exposure 20 seconds—85 per cent. membrane production.

E. Butyric acid exposure $1\frac{1}{2}$ minutes, no membranes produced.

F. Butyric acid exposure 3 minutes, no membranes produced.

G. Butyric acid exposure 5 minutes, no membranes produced.

After butyric acid treatment all were poured into alkaline sea water to stop action of acid, and collected in graduated cylinders. *A*. 0.5 c.c. eggs after treatment; *B*. 0.8 c.c.; *C*. 0.9 c.c.; *D*. 1.1 c.c.; *E*. 0.7 c.c.; *F*. 0.5 c.c.; *G*. 0.4 c.c.

10:30 A.M. began series of washings (Table IV).

It will be noted in this series of eggs that in every case with the exception of lot *C*, fertilization is possible at least to a limited extent, and that in all, fertilizin was still being produced after eight washings, yet none of these eggs possessed a trace of the

TABLE IV.

	A.	*B.*	*C.*	*D.*	*E.*	*F.*	*G.*
10.35 A.M.	10 / 0.5	10 / 0.8	10 / 0.9	10 / 1.1	10 / 0.7	10 / 0.5	10 / 0.4
11.00 A.M.	10 / 0.5	10 / 1.0	10 / 1.5	10 / 1.5	10 / 1.0	10 / 0.7	10 / 0.8
11.30 A.M.	10 / 0.6	10 / 1.2	10 / 1.3	10 / 1.5	10 / 1.5	10 / 0.8	10 / 0.8
12.45 P.M.	10 / 0.5	10 / 1.0	10 / 1.2	10 / 1.4	10 / 0.9	10 / 0.8	10 / 0.7
1.50 P.M.	5 / 0.3	4 / 1.0	5 / 1.1	5·5 / 1.3	4 / 0.9	2.5 / 0.7	1[1] / 0.5
Reaction	No test	4–5 sec.	6–8 sec.	8–9 sec.	6–8 sec.	8–9 sec.	8 sec.
2.25 P.M.	10 / 0.3	10 / 0.9	10 / 1.2	10 / 1.2	10 / 0.9	10 / 0.5	10 / 0.5
Inseminated 2.35 P.M., cleavages	95%	85%	85%	10%	60%*[2]	50%*[2]	50%*[2]
3.15 P.M.	10 / 0.4	10 / 1.0	10 / 1.2	10 / 1.2	10 / 0.8	10 / 0.6	10 / 0.4
4.00 P.M.	2 / 0.4	4 / 0.9	5 / 1.0	5·5 / 1.2	4 / 0.8	2.5 / 0.7	1[1] / 0.5
Reaction	1¼ min.	10–12 sec.	10 sec.	6–7 sec.	9–10 sec.	8–9 sec.	10 sec.

transparent jelly except the control lot. In *C*, eggs to which sperm had been added, only 85 per cent. had been fertilized. There were then 15 per cent. of the eggs still capable of producing fertilizin and we see that a ten-second reaction was given after the eighth washing.[3] Neither is this due to the presence of jelly for this had been dissolved by means of a short butyric acid exposure. In many of the experiments the jelly was destroyed by shaking the eggs a few times in a test tube before the series of washings had begun; the jelly is very easily removed in this way and in all cases where an appreciable number of eggs were not fertilized the agglutination reaction could be obtained.[4]

[1] Eggs 1 part, sea water, 4 parts.

[2] Cleavage was so irregular and cytolysis beginning in so many, that the count is probably far from correct. Only a very small per cent. ever reached the larval stage where even a slight amount of motion was discernible.

[3] In many lots of eggs especially resistent to sperm practically as high an agglutination action was obtained as in eggs easily fertilized. Lack of fertilization is evidently due to physical conditions that prohibit entrance of sperm. When 100 per cent. of fertilizations are obtained no agglutination is found, provided that the time limit of fertilization is complete and also that the eggs have been deprived of their jelly that is saturated with fertilizin liberated from the egg.

[4] It must be emphasized that one should use only freshly prepared sperm sus-

Supernatant fluid of *B*, *E*, and *F* eggs of the experiment was quite decidedly colored by escaping pigment from broken down or injured eggs. In the *B* lot fertilization was much more nearly that of the normal process than was *D*, *E*, *F*, or *G*—approximately 70 per cent. of swimming larvæ were noted in *B*, while only a very small per cent. (10 per cent. to 15 per cent.) were found in the lots that were given a longer butyric treatment; yet we see little difference in the intensity of the agglutination reaction. The tests are complicated by the entrance of the secondary substances diffusing from the eggs. A study of these conditions may reveal striking results. The writer has been entirely unable to produce a curve of fertilizin production that will run parallel to that of fertilization. One fact, however, is to be noted with emphasis. In no case has he ever obtained fertilization of the eggs of *Arbacia* when the fertilizin reaction was negative. Whether this will always hold true remains for further investigation to prove.

3. *Conditions where Fertilization is Absent.*

Since the hypothesis of fertilizin production holds good for all cases of fertilization in the curve where cleavage follows fertilization, the writer was anxious to know if it served equally well as a possible indication of the internal conditions of eggs that would not fertilize. We have seen in these experiments three instances of treatment that prohibit fertilization; penetration of sperm was not prohibited for we have seen in each of the cases of (1) an optimum exposure to butyric acid and subsequent destruction of membranes; (2) prolonged exposure to butyric acid (10 minutes); and (3) exposure to temperature of 35° C. for 10 minutes, that sperm entered in considerable numbers.

Does fertilization then depend upon the presence of fertilizin?

The results of observations on all three conditions answer this in the affirmative. When eggs are given the optimum butyric acid treatment and full membranes are produced fertilizin is not detectable in the supernatant fluid of such eggs after a few washings.

pensions made from dry sperm that has not been standing too long, especially if the original quantity is small. Suspensions should not be older than five minutes or ten minutes at most, if delicate results are desired.

Lillie ('14) has already given proof of this as is shown in his Table VII, page 560. The present results, however, confirm his assertions that wherever fertilizin is absent the fertilization capacity is also entirely lacking. A typical experiment, one of a large number performed, will give the results of the whole series of conditions in which the eggs would not respond to the influence of sperm.

Experiment 65. August 26, 1915.

9:00 A.M., eggs collected, washed and divided into three lots *A*, *B*, and *C*.

A. Exposed to butyric acid for 20 minutes and returned to alkaline sea water to neutralize acid.

B. Exposed to sea water 35° C. for 10 minutes, shaken very gently to free from surrounding jelly.

C. Exposed to butyric acid for 20 seconds, returned to alkaline sea water to stop action of acid. 92 per cent. of eggs produced membranes.

Eggs collected into graduated cylinders and volume of egg noted.

A, 1.1 c.c.; *B*, 1.0 c.c.; *C*, 2.0 c.c.

10:20 began a series of washings. Results given in Table V.

Thus in each of the three cases no cleavages occurred and no fertilizin is being produced. The butyric acid exposure of 20 minutes in this experiment is much longer than necessary to prevent fertilization as was learned later. But in experiments where a much shorter exposure was employed, and a small percentage of cleavages were present, fertilizin was present.

In all instances then in which eggs have been artificially altered in their fertilization capacity we have seen the absolute correspondence of the presence or absence of fertilizin. In the above instances we may use the agglutinin reaction as a test for the capacity of fertilization. The writer does not wish to be understood as maintaining that no other processes are involved in these conditions but in whatever condition we have encountered the egg, if it was found to be actively secreting fertilizin, fertilization has been possible at least to some extent; wherever fertilizin was absent we were never able to obtain fertilization. The correlation is a very close one and one appearing to be of great significance.

TABLE V.

	A.	*B.*	*C.*
10.20 A.M.	25	25	25
	1.1	1.0	2
10.50 "	25	25	"
	1.5	1.5	
11.25 "	"	"	"
11.50 "	"	"	"
12.10 P.M.	"	"	"
Inseminated 12.30 "	No cleavage	No cleavage	No cleavage
12.40 "	25	25	25
	1.5	1.5	2
1.40 "	"	"	"
2.00 "	"	"	"
2.50 "	5	3	10
	1.0	1.0	2.0
Reaction	Negative	Negative	Negative[1]
3.30 P.M.	25	25	25
	1.0	1.0	2.0
4.00 "	"	"	"
4.40 "	5	3	10
	1.5	1.5	2.0
Reaction	Negative	Negative	Negative[2]

VI. DISCUSSION.

In presenting the results obtained during the two previous summers, the writer has employed largely the terminology of the fertilizin hypothesis; no other theory seems capable of offering an explanation of the facts as they have been revealed by experiments. We have seen in Section III. that the lysin theory of fertilization has been entirely disproved by the very material and methods that were used in formulating the hypothesis. On the other hand all the results obtained find a ready explanation in terms of the fertilizin hypothesis. We have previously noted the quantitative aspects of fertilization in the case of the fertilization curve after butyric acid parthenogenesis (Sec. III.). Wherever there is development, certain substances within the egg have been activated either artificially or by a spermatozoön.[3] If the activation has been initiated by a sperma-

[1] Control for freshness of sperm with unknown strength of fertilizin—60 sec. reaction.

[2] Control for freshness of sperm with unknown strength of fertilizin—70 sec. reaction.

[3] If we do not assume that the egg contains all the essentials for development and that these are started in their processes by various activating agents, we have no explanation for parthenogenetic development, normal or artificial.

tozoön it is complete, and bars further activation; but in activation by artificial agents we arbitrarily select the given conditions, and if these are not the optimum conditions complete activation is not obtained. This is evidenced by the further reaction with the sperm, that usually results in very abnormal development. The main question at issue is: Can fertilization be superposed on parthenogenesis?

In the natural history of an egg there appears first a period at which fertilization is not possible, even though sperm enter the eggs. We say such eggs are not mature. Usually also at this period artificial initiation of development is impossible. Conditions are not such as will permit of even a start in the developmental processes. This non-fertilizable condition is followed by changes (maturation) that lead to a condition in which fertilization is the normal behavior. In some eggs this condition is retained but a short while and in others for a longer period. In both cases however the eggs become immune to the effect of sperm shortly after fertilization occurs; they have returned to a non-fertilizable condition.

Lillie has determined that at the time the capacity of fertilization is gained the substance fertilizin is present in very large quantities and that so long as this substance is being liberated, fertilization normally follows the entrance of sperm. When once this process has occurred, further fertilization is absolutely impossible; also fertilizin is no longer being produced by the egg. If this substance is completely washed out of normal, ripe eggs they can not be fertilized.[1]

The studies of E. E. Just ('15) very clearly reveal the loss of some substance that escapes from the egg of *Platynereis* or is changed into an inactive form by an extremely short exposure to sea water. After such an exposure of the eggs, spermatozoa may enter and call forth very slight changes more or less characteristic of normal development, but are yet entirely unable to lead on to cleavage stages and later development. The eggs have returned to a non-fertilizable condition obviously due to the absence of some substance which must be present if the fertilization reaction is to be carried out completely.

[1] Lillie, '14, —

In the experiments here presented we have clearly depicted a set of conditions, each somewhat different from the others, that reveal gradations from the normal condition to the boundary line of the possibilities, and even to a complete absence of the fertilization reaction. We have no indication that the processes of activation resulting in complete membrane production are different, in any of the different methods by which it may be called out. But two things we see in common (1) the non-fertilizible condition of the egg—whether produced by the addition of sperm or as a result of artificial stimulation and (2) the absence of fertilizin. While eggs are in this non-fertilizable condition sperm may enter them in great numbers, but are not more efficient in producing further developmental phenomena than in the case of unripe eggs.

We see further that exposures to the same substances used to call out membranes can be conducted so that no membranes are produced (indication of quantitative phase of activation) and that the addition of sperm does permit a certain amount of development; but this development is usually very widely separated from the normal. In all instances where treatment with these agents permits any effect of the spermatozoön at all, fertilizin has been present in quantities sufficiently large to be detected by its property of agglutinating sperm.

Also by exposing ripe eggs to butyric acid, both for the optimum time to produce membranes, and for a prolonged exposure, conditions have been produced in which the eggs appear normal but yet addition of sperm does not result in development. We have determined that sperm penetrates these eggs but are nevertheless ineffective; fertilization is entirely lacking. Also in these instances we have always encountered an absence of fertilizin. Thus it is fairly well established that wherever fertilization is possible fertilizin is present. With this so thoroughly indicated the answer to our question—Can fertilization be superposed upon parthenogenesis—is very easily answered.[1] *When an*

[1] It appears that some artificial agents have the power of calling forth a certain amount of development without inducing cortical changes of the egg. Hypertonic sea water, as shown by Loeb, in certain instances may cause development but it is not always capable of doing so. Just what conditions are necessary for its action are still not well understood and I shall defer any discussion of its effects until a later date.

exposure of eggs to artificial agents has as a result, the imitation of the effect of a spermatozoön in that full membranes are produced around the egg, or when activation of fertilizin has been completed, superposition of fertilization is impossible. If the activation has been completed all fertilizin has been rendered inactive and a spermatozoön has no effect upon the condition of the egg leading to further development.

The spermatozoön, it seems, to be able to produce its normal effect, must enter into the developmental reactions at the beginning. Whether some substance from the sperm forms a union with fertilizin, that paves the way for a normal reaction, is as yet problematical, but it is evident from these experiments and others that sperm must enter the egg in the normal way if it is to be normally effective.

Thus fertilization appears very decidedly, to be a continuous process each step leading to the next. If the first steps are artificially initiated, spermatozoa entering, either do not become effective at all or in doing so normal processes deviate so decidedly from their natural course that developmental results usually depart very decidedly from the normal. If the first steps leading to fixation of fertilizin are artificially initiated and completed, insemination, even if the spermatozoon penetrates the egg, has no effect. If these first steps are incomplete and some fertilizin remains unbound, partial fertilization may result in all degrees, as evidenced by the abnormal course of cleavage and development.

VII. Summary.

1. Normal *Arbacia* eggs exposed to butyric acid (50 c.c. sea water + 2.8 c.c. *N*/10 butyric acid) and subsequently fertilized, reveal a curve of fertilization falling from the normal reaction to the optimum exposure to butyric acid for membrane production: rising from the optimum exposure (20 sec.) to an over-exposure of $2\frac{1}{2}$–3 minutes; and again falling off until no further fertilization is possible—about 10 minutes.

2. Eggs having been exposed for the optimum time to butyric acid, have acquired an unfertilizable condition. If the membranes are completely removed, sperm enter the eggs but do not lead to development. The eggs, though sperm have entered them, are not fertilized.

3. Sperm having entered these eggs appear only as foreign bodies. They do not cause the production of asters, nor are cleavage spindles produced.

4. The lysin theory of fertilization is found inadequate to explain the results.

5. Eggs over-exposed to butyric acid are still capable of a certain amount of fertilization, though the process is usually very abnormal. No membranes are produced. Polyspermy is extremely frequent.

6. Exposure to butyric acid for ten minutes leads to a non-fertilizable condition of the egg. Sperm enter but are non-effective and non-effected.

7. Eggs subjected to a temperature of 35° C. for 10 minutes are rendered non-fertilizable. Sperm enter the eggs, part disintegrating in fragments, part forming large clear vesicles and part apparently remain unaffected.

8. Where fertilization is possible fertilizin has always been found present. Where fertilization is not possible fertilizin has never been found.

9. The quantitative aspect of fertilization is very definitely indicated.

10. Superposition of fertilization upon parthenogenesis, where activation has been complete (indicated by full membrane production and absence of fertilizin) is impossible.

Where activation by parthenogenetic agents has been only partially completed, partial fertilization is yet possible, but development is usually abnormal.

April 15, 1916.

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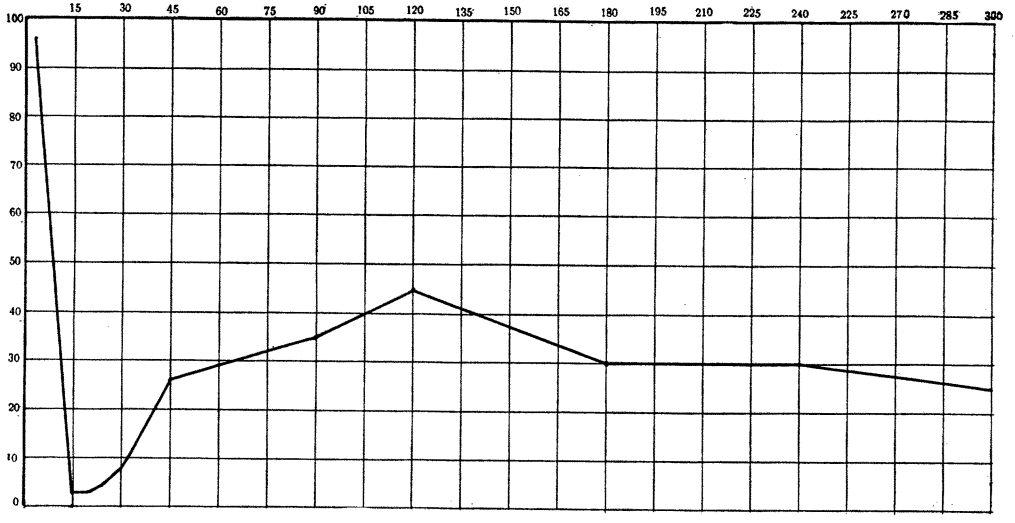
EXPLANATION OF PLATE.

FIG. 1*a*. Curve of fertilization following butyric acid treatment. The ordinates represent percentages of fertilization as measured by cleavages, and the abscissae the length of exposure to butyric acid in seconds. The curve is completed by Fig. 1*b*.

FIG. 1*b*. Completion of curve of fertilization following butyric acid treatment. For first part of curve see Fig. 1*a*.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100 90 80 70 60 50 40 30 20 10 0

Fig. 1*a*.

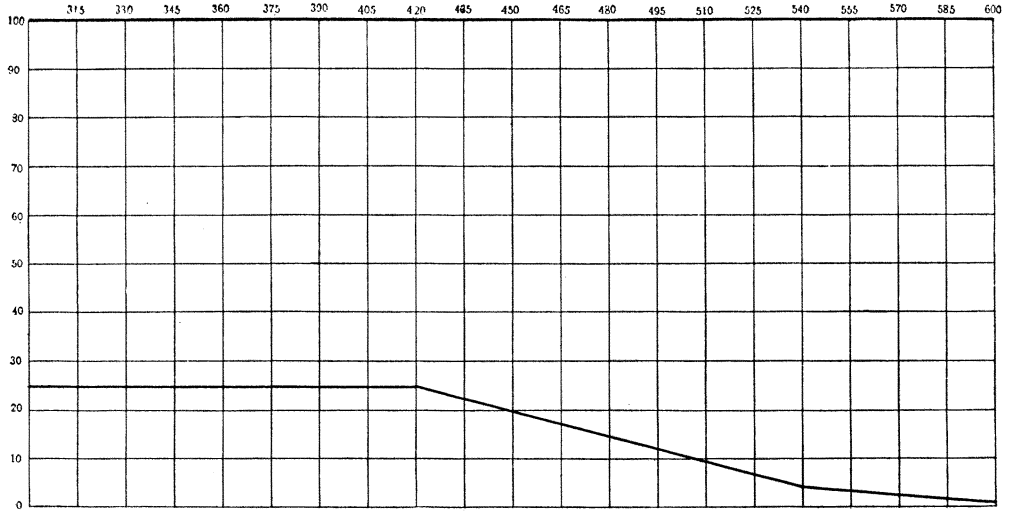
315 330 345 360 375 390 405 420 435 450 465 480 495 510 525 540 555 570 585 600
100 90 80 70 60 50 40 30 20 10 0

Fig. 1*b*.